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Loss of heat acclimation capacity could leave subterranean specialists highly sensitive to climate change

Short title: Acclimation capacity in subterranean specialists

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Abstract

Physiological traits are key in determining the vulnerability of narrow range, highly specialised animals to climate change. It is generally predicted that species from more stable environments possess lower thermal tolerance breadths and thermal plasticity than those from more variable habitats – the so-called ‘climatic variability hypothesis’. However, evolutionary trade-offs between thermal breadth and its plasticity are also seen in some taxa, and the evolution of thermal physiology remains poorly understood. Subterranean environments are excellent systems for exploring these issues, being characterized by stable climatic conditions, with environmental variability increasing predictably from deep to shallow habitats. Acclimation capacity will be fundamental in determining the sensitivity of subterranean species to climate change, since they have poor dispersal capacity and limited possibility to exploit thermally different microhabitats in the uniform cave environment. We assessed critical thermal maximum (CT_{max}) and short-term heat acclimation capacity in three related beetles (Leiodidae: Leptodirini) with differing degrees of specialisation to the subterranean environment (deep, shallow and facultatively subterranean, respectively) and therefore exposed to contrasting thermal variability in nature. Only the facultative subterranean species showed any acclimatory capacity, also having the highest CT_{max} across the taxa studied. However, this species might experience the highest thermal stress in its habitat under climate change. The studied subterranean specialists will be poorly able to cope physiologically with temperature increase, but in contrast exposed to lower magnitude and rate of warming. Our results fit the climatic variability hypothesis, suggesting that adaptation to cave conditions has selected against the retention of acclimation mechanisms. We show that the pathways that determine vulnerability of subterranean species to climate change depend on their degree of specialisation to deep subterranean environments. This information, combined with evaluation of exposure to climatic changes at their present locations, is fundamental in identifying species or populations at greatest risk.

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26 **Keywords:** critical thermal maximum, physiological plasticity, thermal tolerance, climate
27 variability hypothesis, subterranean environment, conservation, cave beetles, climate change

Review Copy

28 Introduction

29 It is broadly recognised that physiological data may contribute directly to conservation in the
30 face of climate change and advance the field of conservation physiology (Wikelski & Cooke,
31 2006; Cooke & O'Connor, 2010; Cooke *et al.*, 2013; Evans, 2015). Indeed, accurate assessments
32 of species vulnerability to climate change require integrated measurements of its different
33 determinants, including exposure (the magnitude and change of climatic change) and factors of
34 organisms' sensitivity at the individual level, such as the physiological tolerance to
35 environmental change (Williams *et al.*, 2008; Foden *et al.*, 2019). In this context, the acclimation
36 capacity of organisms determines their ability to track changing environmental conditions and is
37 of fundamental importance, especially for species that are unable to respond to climate change
38 by other means (e.g. range shifts, behavioural adjustments or evolutionary adaption) (Stillman,
39 2003; Seebacher, White, & Franklin, 2015). Thermal acclimation occurs when individuals adjust
40 their physiology to compensate for the effects of temperature variation, and may ultimately
41 extend critical thermal limits for performance and survival (e.g. Da Silva, Riginos & Wilson,
42 2019).

43 Despite multiple hypotheses proposed to understand how thermal plasticity varies across taxa
44 (see Rohr *et al.* (2018) for a recent review), empirical studies suggest that acclimation responses
45 are highly context-dependent. According to the climatic variability hypothesis (CVH) (Stevens,
46 1989), species from more stable environments are predicted to have lower thermal tolerance (i.e.
47 narrower tolerance breadths and less acclimation capacity) than those from more variable
48 habitats (e.g. Feder, 1978; Tomanek, 2008; Magozzi & Calosi, 2015; Shah, Funk, & Ghalambor,
49 2017; Markle & Kozak, 2018), but such a pattern has not always been supported (Seebacher *et*
50 *al.*, 2015). Furthermore, evolutionary trade-offs may constrain the evolution of the different
51 components of thermal tolerance, so that species that have evolved the greatest thermal limits

(particularly upper ones) may have done so at the expense of the plasticity of such limits and therefore show limited acclimation capacity (e.g. Stillman, 2003).

Testing between such alternatives is challenging, given the multiple intrinsic and extrinsic factors that affect the range of temperature actually experienced by organisms. For the same reason, teasing apart the relative importance of the different drivers of vulnerability to climate change can be challenging. Climatically stable and isolated systems are excellent models to minimize confounding effects and focus on physiology in a climate change context. This is the case with subterranean habitats, where environmental conditions are more stable and homogeneous than at the surface, limiting the possibility of behavioral adjustments of their inhabitants through differential microhabitat use (Sánchez-Fernández *et al.*, 2018). The dispersal potential is also extremely reduced for most subterranean specialists (Juan & Emerson, 2010; Rizzo *et al.*, 2017). Moreover, low genetic variability (Juan *et al.*, 2010), low reproductive rates and long life cycles (Voituron *et al.*, 2010) may constrain rapid evolutionary change in such taxa (Sánchez-Fernández *et al.*, 2016; Mammola *et al.*, 2019b). Therefore, as with species in many other isolated ecosystems (e.g. mountain summits or islands), they rely strongly on physiological adjustments to cope with environmental changes, even though the magnitude and rate of such changes in subterranean habitats is moderate compared to the surface (Domínguez-Villar *et al.*, 2015; Pipan *et al.*, 2018). Deep subterranean habitats typically experience very stable climates (but see Trontelj, Borko, & Delić, 2019) and within caves, such stability decreases markedly from inner to outer sections (Cigna, 2002; Badino, 2010; Pipan *et al.*, 2010), presenting an ideal setting in which to explore the relationship between thermal tolerance and climatic variability and test macrophysiological hypotheses (Gaston *et al.*, 2009; Sánchez-Fernández *et al.*, 2018; Mammola *et al.*, 2019b).

The rarity of many subterranean taxa (Sánchez-Fernández *et al.*, 2018; Trontelj *et al.*, 2019) and difficulties of access for sampling (Raschmanová *et al.*, 2018; Castaño-Sánchez, Hose, &

Reboleira, 2020; Mammola, *et al.*, 2019a), have limited research on subterranean species to date. In terms of thermal tolerance, most of the data on subterranean organisms come from studies conducted on a handful of species and have generally shown they have a high thermal sensitivity: upper lethal limits are usually not higher than 23°C (e.g. Issartel *et al.*, 2005; Rizzo *et al.*, 2015; Pallarés *et al.*, 2019) and studies based on molecular biomarkers indicate that thermal stress onsets at lower temperatures (Mermillod-Blondin *et al.*, 2013; Pallarés *et al.*, 2020). Several studies have also suggested that the degree of specialisation to the subterranean environment is negatively correlated with thermal tolerance breadth (Lencioni, Bernabò & Latella, 2010; Bernabò *et al.*, 2011; Novak *et al.*, 2014; Raschmanová *et al.*, 2018; Mammola *et al.*, 2019c), broadly supporting the CVH. Whether physiological plasticity is reduced in the process of specialisation to subterranean life and how it could affect species sensitivity to climate change remains an open question, however, since most studies have ignored acclimation capacity (but see Rizzo *et al.*, 2015; Pallarés *et al.*, 2019, 2020). In addition, such studies have often involved disparate, unrelated species, sometimes from localities with very different climates, making meaningful comparisons difficult. Efforts to increase our knowledge on the thermal tolerance of subterranean fauna are needed, as they represent an important and typically endemic component of the global animal biodiversity, but still largely neglected in conservation programs.

In Leptodirini cave beetles (Coleoptera, Leiodidae), one of the most extreme modifications associated with underground colonisation is a reduction in the number of larval instars in the life cycle, likely as an adaptation to a resource-poor environment (Cieslak, Fresneda, & Ribera, 2014a, b). Species that can live in deep forest litter or the upper layers of fractured soil (MSS, *Milieu Souterrain Superficiel* (Juberthie, Delay, & Bouillon, 1980a,b, 1981; Mammola *et al.*, 2016)), i.e., not necessarily confined in caves, typically have the standard life cycle of Coleoptera, with three larval instars (Minelli & Fusco, 2013). Deep or shallow subterranean

specialists (true troglobionts *sensu* Sket (2008)) may show a reduction to two or -in the most extreme specialisation- one larval instar. Therefore, the number of larval instars in these species, clearly associated with the degree of subterranean specialisation, can be used as an indirect proxy for the degree of thermal stability species experienced in their evolutionary history. Here we assess critical thermal maximum (CT_{max}) and short-term heat acclimation capacity in three related Leptodirini taxa from areas with similar climatic histories but with life cycles of one, two and three larval instars respectively, reflecting different degrees of specialisation to the subterranean environment and hence exposed to contrasting thermal variability. We explore whether support for the CVH in subterranean taxa (Latella, Bernabò & Lencioni, 2008; Lencioni *et al.*, 2010; Novak *et al.*, 2014; Raschmanová *et al.*, 2018; Mammola *et al.*, 2019c) also holds for thermal plasticity. Under the CVH, both CT_{max} and acclimation capacity are expected to decrease with increasing subterranean specialisation. However, if trade-offs between thermal limits and plasticity have shaped the evolution of thermal tolerance in these taxa, such a relationship with subterranean specialisation might not be so evident for acclimation capacity (i.e., species living in the more variable habitats could tolerate higher temperatures but show little thermal plasticity). Extending these general hypotheses to the subterranean environment can provide important insights into the evolution of thermal physiology associated with the process of underground specialisation, as well as relevant information for the conservation of specialist animal taxa with limited dispersal capabilities.

Material and methods

Study species and collection

The study species belong to monophyletic lineages of the beetle tribe Leptodirini; the Pyrenean (*Speonomites crypticola* and *Bathysciola rugosa*) and Cantabrian clades (*Speonomidius crotchii*) (Salgado, Blas, & Fresneda, 2008; Ribera *et al.*, 2010). Rearing experiments revealed that *S.*

126 *crotchi* has a life cycle of two larval instars (Jeannel, 1911; Glaçon, 1955), whilst *S. crypticola*
 127 and *B. rugosa* were inferred to have one and three larval instars, respectively, based on robust
 128 ancestral state reconstructions (Cieslak *et al.*, 2014b). Two of the species are obligate cave
 129 inhabitants only found in deep subterranean habitats (*S. crypticola*) or in both deep and shallow
 130 subterranean environments (*S. crotchi*), whilst *B. rugosa* is a facultative subterranean species,
 131 most commonly found in deep forest litter, always in dark conditions (Salgado *et al.*, 2008).

132 Live adults were collected in different sites within the same geographical region (Northern
 133 Spain), with broadly similar mean annual surface temperatures (Table 1), which are known to
 134 closely approximate the constant temperatures recorded inside caves (Moore & Sullivan, 1964;
 135 Smithson, 1991; Badino, 2004, 2010). Therefore, all species live under similar average climatic
 136 conditions but occupy habitats with different daily and seasonal climatic variability. The two
 137 subterranean species can be assumed to be exposed to an annual variation of a few degrees ($\leq 4^{\circ}$
 138 C) around mean annual surface temperatures, which is the typical thermal range in caves
 139 (Badino, 2010; Mammola & Isaia, 2016). The facultative subterranean species can be assumed to
 140 be exposed to a maximum annual variation between 8 – 24.4° C (minimum temperature of the
 141 coldest month and maximum temperature of the warmest month at the surface in its locality,
 142 respectively, obtained from Worldclim v. 1.4 database (<http://www.worldclim.org>; see Table 1).
 143 Projected temperatures for 2070 under the Representative Concentration Pathway (RCP) 4.5 are
 144 also shown in Table 1.

145 Specimens of *S. crypticola* and *S. crotchi* were collected by hand in the caves indicated in Table
 146 1, whilst specimens of *B. rugosa* were collected in forest litter near the entrances of Cave Orobe.
 147 The specimens were transported to the laboratory in a portable fridge with substratum from the
 148 cave and moss to retain humidity (ca. 90% RH). In the laboratory, they were maintained for two
 149 days prior to experiments in closed plastic containers (10 x 15 x 10 cm) with a plaster layer (1
 150 cm), in controlled-temperature incubators (Radiber ERF-360, Radiber S.A, Barcelona, Spain) at

the approximate mean temperature of their habitats (*S. crypticola* 11°C; *S. crotchii* 9°C; *B. rugosa* 12°C), i.e. control temperatures hereafter. Maintenance of high humidity (> 90% RH) throughout the experiments was ensured by adding wetted tissue paper to experimental containers daily and placing trays with water inside the incubators. Temperature and humidity inside the containers were monitored with HOBO MX2301 dataloggers and remained quite constant, with very small variations ($\pm 0.5^\circ\text{C}$ and $\pm 10\%$ RH). Food was provided *ad libitum* with freshly frozen *Drosophila melanogaster*.

Upper thermal limits and acclimation capacity

Specimens were acclimated at either a control temperature (see above) or at 20°C for 10 days (N = 10-13 individuals per treatment), with all other conditions (humidity, food etc.) as described before. These acclimation temperatures were chosen since all subterranean leiodids studied to date can survive relatively long-term exposure (>7 days) at 20°C, but this is also close to their upper lethal limits (Rizzo *et al.*, 2015; Pallarés *et al.*, 2019, 2020), and it is well established that insects typically undergo acclimation in response to stressful sublethal temperatures (e.g. Lachenicht *et al.*, 2010; Nyamukondiwa & Terblanche, 2010; Terblanche *et al.*, 2011; Sgrò, Terblanche, & Hoffmann, 2016). After acclimation, we estimated heat coma temperature (HCT) for each individual. This was defined as the temperature at paralysis prior to death, preceded by spasmodic movements of legs and antennae, and is a typical response used to assess critical thermal maximum (CT_{max}) in insects (see Vannier, 1994; Lutterschmidt & Hutchison, 1997; Chown & Nicolson, 2004). HCT was measured using a dynamic, ramping method (Lutterschmidt & Hutchison, 1997; Terblanche *et al.*, 2007), with a heating rate of 1°C min^{-1} and infrared thermography. CT_{max} measured with such a fast ramping rate tend to be overestimated (Terblanche *et al.*, 2007) and cannot be used to directly extrapolate actual thermal limits, but this method provides a robust comparative estimate of the relative thermal tolerance amongst related species and is the most commonly used in thermal tolerance assays on ectotherms (Bennett *et al.*,

2018). Furthermore, infrared thermography has proven to be a highly sensitive technique to measure CT_{\max} in insects (Botella-Cruz *et al.*, 2016; Carbonell *et al.*, 2016; Gallego *et al.*, 2016; Gallego, Verdú, & Lobo, 2018). Specimens were dried on blotting paper and glued dorsally on a ceramic plate using nontoxic glue (ErichKrause) to prevent escape during the trial. The plate was then placed in a programmable BINDER MK53 incubator (BINDER GmbH, Tuttlingen, Germany) in which the temperature was increased $1^{\circ}\text{C min}^{-1}$, starting at the corresponding acclimation temperature. The incubator was coupled with an infrared thermographic camera (FLIR SC305) to record body temperature of the specimens, from which HCT were obtained. High quality images were also recorded with a video camera (Sony DCR-DVD110E, Sony Co., Tokyo, Japan) to determine the moment of paralysis (cessation of movement of legs and antennae) of each individual. Thermal images were analysed with the software ThermoCAM Researcher Professional 2.10 (FLIR Advanced Thermal Solutions; ATS; Croissy-Beaubourg, France).

Statistical analyses

We performed a two-way ANOVA with HCT as the response variable and species, acclimation temperature (a factor with two levels: control temperature (see above) or 20°C) and their interaction as predictors, to compare HCT and short-term acclimation capacity across species and assess whether these traits are related to the degree of subterranean specialisation. Pairwise comparisons were made with Bonferroni-adjusted post-hoc tests. Significance level was set at 0.05. Data conformed to a normal distribution so no transformation was required. Normality and homoscedasticity assumptions of model residuals were also validated by graphical inspection (Zuur *et al.*, 2009). All statistical analyses were conducted in R v.3.6.1 (R Core Team, 2019) using default packages and *phia* (De Rosario-Martinez, 2015).

Results

Heat coma temperatures (HCTs) ranged from 34.6 – 38°C across the studied species and acclimation treatments (Figure 1). HCTs differed significantly amongst species (ANOVA $F_{2, 52} = 21.963$, $P < 0.001$), being lower in the one-larval instar, deep subterranean species (*S. crypticola*) than in the other two, less specialised taxa investigated (all Bonferroni corrected P s < 0.001 , Table 2). There was also a significant effect of acclimation temperature on HCT (ANOVA $F_{1, 52} = 18.357$, $P < 0.001$), but species differed significantly in their response to acclimation (ANOVA species x temperature interaction $F_{2, 52} = 3.564$, $P = 0.035$).

The deep subterranean specialist, *S. crypticola*, had a significantly lower HCT than the two-instar, shallow subterranean *S. crotchii* when acclimated at control temperatures, and lower than either of the other two species following acclimation at 20°C (all Bonferroni corrected P s < 0.001 , Table 2; Fig.1). Only the species with the least specialisation to subterranean environments, *B. rugosa* (three larval instars), showed acclimation capacity. In this species, HCT significantly increased after exposure at 20°C (Bonferroni corrected $P < 0.001$, Table 2; Fig. 1), with a difference of 1.2°C between the mean HCT of the control and 20°C treatment. The more specialised subterranean taxa lacked acclimation ability because no differences were found in HCT between acclimation treatments (Bonferroni corrected P s > 0.05 , Table 2; Fig. 1).

Discussion

Among the three studied subterranean species, the most specialised to the subterranean environment and hence living under the more stable climatic conditions, *S. crypticola*, showed the lowest HCT values. Such finding in relation to the upper thermal limit fits the general predictions of the CVH, as seen in other subterranean groups for both lower (Latella *et al.*, 2008; Lencioni *et al.*, 2010; Novak *et al.*, 2014) and upper thermal limits (Raschmanová *et al.*, 2018; Mammola *et al.*, 2019c). The main novelty of our study is that the CVH is tested by comparing not only thermal limits, but also thermal plasticity, something largely ignored for subterranean

224 taxa to date. Again, in agreement with the CVH, the more specialised subterranean taxa (*S.*
 225 *crypticola* and *S. crotchi*, both obligate subterranean) lacked acclimation ability at the
 226 acclimation temperatures and heating rates tested here; whilst *B. rugosa*, the facultative
 227 subterranean species exposed to more fluctuating daily and seasonal temperatures, significantly
 228 increased HCT after acclimation at the higher temperature. As well as a positive acclimation
 229 response, *B. rugosa* showed the highest HCT values across the three species after acclimation at
 230 20°C. Therefore, there is not apparent evolutionary trade-off between CT_{max} and its short-term
 231 plasticity in these species, as reported in a clade of aquatic beetles (Calosi, Bilton & Spicer,
 232 2008), but in contrast to patterns seen in other taxa such as intertidal porcellanid crabs (Stillman,
 233 2003). As suggested by Calosi *et al.* (2008), the mechanisms and constraints underlying thermal
 234 limits and acclimation capacity appear to be taxon and context specific.

235 A lack of thermal acclimation capacity has also been reported for other subterranean groups,
 236 such as groundwater crustaceans (Di Lorenzo & Galassi, 2017), and previous work on
 237 Leptodirini suggests that the physiological mechanisms to cope with heat stress are rather
 238 inefficient in specialised subterranean species compared with their less specialised relatives. For
 239 example, Bernabò *et al.* (2011) showed that the intensity of the heat shock response was
 240 negatively correlated with the degree of adaptation to the cave environment. Pallarés *et al.*
 241 (2020) found that an obligate subterranean species of the clade studied here failed to acclimate
 242 when exposed to a fixed stressful temperature. Such inability to acclimate was coupled with
 243 oxidative stress and shifts in the activity of key enzymes at sublethal temperatures. The
 244 physiological mechanisms underlying thermal acclimation capacity in ectotherms are
 245 energetically costly processes (Krebs & Loeschcke, 1994; Monaghan, Metcalfe, & Torres, 2009;
 246 Tomanek, 2010) that could have been selected against during the evolution of specialisation to
 247 deep subterranean habitats. In these environments, with limited resources and highly stable
 248 climatic conditions, both temporally and spatially (Badino, 2010; Howarth & Moldovan, 2018),

249 traits that do not affect fitness could be lost or reduced either by neutral evolution or indirect
250 natural selection favouring energetic economy (Culver & White, 2005, Ribera *et al.*, 2018).
251 Extreme modifications of thermal physiology have been found in organisms inhabiting other
252 highly stable habitats, such as the absence of a heat shock response in Antarctic notothenioid fish
253 (Somero, 2005). The greatest modifications of thermal physiology in subterranean taxa, resulting
254 in lower thermal limits and poor acclimation capacity, may be expected in those lineages that
255 colonised subterranean habitats longer ago during their evolutionary history. This is the case of
256 Western Mediterranean Leptodirini, which were apparently already adapted to subterranean life
257 by the Early-Mid Oligocene, some 34-28 MYA (Ribera *et al.*, 2010; Cieslak, *et al.*, 2014b).
258 Accordingly, other specialised deep subterranean species of these beetles are also likely to lack
259 acclimation capacity and have similar CT_{max} than the one-larval instar species studied here (*S.*
260 *crypticola*). In contrast, deep subterranean species in lineages that colonised subterranean
261 habitats more recently may retain greater heat tolerance and acclimation capacity from their less
262 specialised or surface-dwelling ancestors, as previously suggested (Pallarés *et al.*, 2019). Further
263 experimental data on thermal limits and acclimation for several lineages that have independently
264 colonised the subterranean environment would be invaluable in testing this evolutionary
265 hypothesis. Unfortunately, the difficulties in accessing subterranean habitats, of collecting
266 sufficient number of specimens for experimental studies and maintaining and rearing
267 subterranean organisms in the laboratory (Raschmanová *et al.*, 2018; Castaño-Sánchez *et al.*,
268 2019; Mammola *et al.*, 2019a) impose serious limitations when conducting comparative studies
269 on cave-dwelling species. Therefore, despite its relatively modest taxonomic coverage, our study
270 represents an important milestone in understanding physiological evolution of subterranean
271 invertebrates.

272 Overall vulnerability to climate change in subterranean species will depend mainly on their
273 thermal sensitivity and the magnitude and rate of climate change in their habitats, as range shifts

274 through dispersal will be largely limited. Both aspects (sensitivity and exposure to climate
 275 change) will in turn be determined by the degree of subterranean specialisation, but in opposite
 276 directions. Here we show that subterranean species have higher thermal sensitivity than surface
 277 ones, something that has been generally assumed but rarely tested. The HCT of the three species
 278 studied are markedly lower than the average value of CT_{max} ($45.5 \pm 1.0^{\circ}C$, $N = 40$) recorded
 279 experimentally in a range of arthropods at the same heating rate (Bennett *et al.*, 2018). We also
 280 show that thermal sensitivity increases with subterranean specialisation. In deep subterranean
 281 Leptodirini, experiments under relatively long-term exposure, which may better represent natural
 282 conditions, have shown that heat injury at the biochemical level occurs at $20^{\circ}C$ (Pallarés *et al.*,
 283 2020) and survival is not possible above $23^{\circ}C$ (Rizzo *et al.*, 2015). Such upper lethal limits seem
 284 to be highly conservative across deep subterranean specialist species of this clade, irrespective of
 285 the current and historical temperature of their habitats (Rizzo *et al.*, 2015). Likewise, the lack of
 286 acclimation observed here in *S. crypticola* and *S. crotchi* appears to be mirrored in related
 287 species with similar specificity to subterranean habitats (as discussed above). In contrast,
 288 changes in the underground climate will be delayed compared to those experienced at the surface
 289 (Fejér & Moldovan, 2013; Domínguez-Villar *et al.*, 2015) and will occur primarily in the cave
 290 sections closest to the surface and in superficial subterranean habitats (Culver & Pipan, 2014),
 291 such as small cavities in the uppermost karst layers, deep soil and litter strata, surface cracks and
 292 fissures (Badino, 2004; Mammola, Goodacre, & Isaia, 2018). In a proximate climate change
 293 scenario, the projected annual mean surface temperature apparently will not reach
 294 physiologically stressful levels for the two obligate subterranean species studied here (Table 1).
 295 However, this cannot be generalized, as some coastal or southern populations of specialist cave
 296 invertebrates in the Mediterranean Basin are currently living at temperatures close to their lethal
 297 limits (e.g. Sánchez-Fernández *et al.*, 2016; Pallarés *et al.*, 2019) and the same could occur in the
 298 tropics (Zeh *et al.*, 2012). As the possibilities for behavioural thermoregulation through

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5 300 considering the limited capacity to physiologically adjust to rising temperature observed here,
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8 301 these habitats may become physiological and evolutionary traps for some of their obligate
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10 302 inhabitants (Mammola *et al.*, 2019b). Compared to species confined to caves, those occupying
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12 303 forest litter or the MSS, like *B. rugosa*, may cope better with warming, because they are able to
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14 304 increase thermal limits via acclimation and exploit a wider range of microhabitats. In this case,
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16 305 the magnitude and rate of warming could be the key determinant of their vulnerability to climate
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18 306 change, as these species are more exposed to the thermal fluctuations of the surface, where
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20 307 maximum temperatures could reach physiologically stressful values in a proximate future (e.g.
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22 308 28.5°C in the localities where *B. rugosa* was collected).
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27 309 In summary, our results stress the need to account for physiological constraints such as thermal
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29 310 sensitivity and acclimation capacity if we are to accurately forecast the impacts of global change
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31 311 on specialised invertebrates with low dispersal ability. This information, combined with
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33 312 evaluation of exposure to climatic changes in occupied locations, can greatly assist in identifying
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35 313 species or populations at higher risk. We show that in the case of the subterranean environment,
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37 314 the relative importance of factors determining overall vulnerability may differ depending on the
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39 315 degree of habitat specialisation. As many authors have already urged (e.g. Sánchez-Fernández *et*
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41 316 *al.*, 2018; Mammola *et al.*, 2019a,b; Castaño-Sánchez *et al.*, 2020), it is necessary to increase
42
43 317 research, monitoring and conservation efforts on this unknown, fragile and valuable component
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45 318 of global biodiversity.
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Tables

Table 1. Information on collection sites and their current and projected temperature.

Species	No instars	Locality	Province	Mean annual Temperature (°C) ^a		Annual Temperature range (°C) ^{a,b}	
				Current	2070	Current	2070
<i>Speonomites crypticola</i>	1	Cave Forat Negre	Lleida	10.4	11.8	-2 – 23.1	-0.8 – 28.0
<i>Speonomidius crotchi</i>	2	Cave Arrikutz	Guipuzcoa	11.1	14.9	3.4 – 24.6	5.2 - 28.4
<i>Bathysciola rugosa</i>	3	Cave Orobe	Navarra	12	13.6	8 – 24.4	26.8 – 28.5

^aSource: Worldclim v. 1.4 database (<https://www.worldclim.org/>), 30 second spatial resolution.

Future projected temperatures in 2070 were obtained for the Representative Concentration Pathway 4.5 and by averaging 17 different Global Circulation Models.

^bMinimum temperature of the coldest month – Maximum temperature of the warmest month

Table 2. Results of post-hoc tests (with Bonferroni-adjusted P-values) to compare heat coma temperature i) among species, ii) between acclimation treatments (C: control vs 20°C) within each species and iii) among species within each acclimation treatment.

	Value	df	Sum of Sq	F	P
<i>B. rugosa</i> - <i>S. crotchi</i>	-0.088	1	0.073	0.220	1
<i>B. rugosa</i> - <i>S. crypticola</i>	1.047	1	8.698	26.125	<0.001
<i>S. crotchi</i> - <i>S. crypticola</i>	1.135	1	13.206	39.668	<0.001
Residuals		52	17.312		
<i>B. rugosa</i> (C - 20)	-1.336	1	6.643	19.953	<0.001
<i>S. crotchi</i> (C - 20)	-0.446	1	1.294	3.886	0.162
<i>S. crypticola</i> (C - 20)	-0.360	1	0.548	1.646	0.615
Residuals		52	17.312		
C (<i>B. rugosa</i> - <i>S. crotchi</i>)	-0.532	1	1.400	4.206	0.272
C (<i>B. rugosa</i> - <i>S. crypticola</i>)	0.560	1	1.327	3.985	0.307
C (<i>S. crotchi</i> - <i>S. crypticola</i>)	1.091	1	6.335	19.030	<0.001
20 (<i>B. rugosa</i> - <i>S. crotchi</i>)	0.356	1	0.577	1.732	1
20 (<i>B. rugosa</i> - <i>S. crypticola</i>)	1.534	1	8.784	26.385	<0.001
20 (<i>S. crotchi</i> - <i>S. crypticola</i>)	1.178	1	6.871	20.638	<0.001
Residuals		52	17.312		

df: degrees of freedom, Sum of Sq: sum of squares

Figure legends

Figure 1. Mean \pm s.e.m heat coma temperatures measured after acclimation at a control temperature (*S. crypticola* 11°C; *S. crotchi* 9°C; *B. rugosa* 12°C) or 20°C. Significant differences between acclimation treatments within each species ($P < 0.05$ in post-hoc tests) are indicated with asterisks.

Figures

